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BY

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NEUROTOXOID INTERFERENCE WITH TWO HUMAN STRAINS OF POLIOMYELITIS IN RHESUS MONKEYS*

Since 1949, an investigation of the interfering effect of neurotoxoids** in poliomyelitis has been in progress in this department. Originally, the rationale was 2-fold: (1) Venom neurotoxins have been reported capable of penetrating the blood-brain barrier and of producing histopathologic changes in motoneurons;[1,2] (2) The experiences of several investigators with the virus of poliomyelitis suggested it was suitable for study since unrelated viral agents have been used successfully as interfering agents.[3,4,5,6] However, in each instance of poliomyelitis "blockade", it had been necessary to have the interfering agent present as infective virus particles.[6] The primary point of interest was the possibility that a non-propagating, non-toxic substance might retain interfering capacity for the poliomyelitis virus without producing irreversible damage to the cells involved. The results have clearly demonstrated the phenomenon of neurotoxoid interference.

Various studies have been made, but the present communication is limited to results obtained when the neurotoxoid was introduced 24 hours after intracerebral infection with 2 strains of human poliomyelitis (Brunhilde and Lansing).

Materials and Methods. Neurotoxoids studied have been those made from *Naja naja* (Indian Cobra), *Naja flava* (Cape Cobra), tetanus and diphtheria*** toxins. The major effort has been expended on detoxicated *Naja flava* venom. A complete report will be published concerning the development of satisfactory procedures for detoxication.[7] Various methods of detoxication have been studied, including heating, formalinization, and oxidation by hydrogen-peroxide-copper systems and ozone. A standard procedure for detoxicating 1% venom by the use of 0.2% formalin, 2% hydrogen peroxide (28% strength) and traces of copper sulfate and catalase has been developed permitting rapid detoxication to a degree shown to be desirable by mouse bioassay and

*This investigation was supported in part by grants-in-aid from Eli Lilly & Co., Indianapolis, Ind., the Mary Schuck Smith Fund, and Mt. Sinai Hospital, Miami Beach, Fla. Grants-in-aid from the following individuals also contributed to the program: Mrs. Margaret D. Boegner, Mrs. Frederick E. Guest, Mrs. Douglas MacCrary, Henry B. Martin, Mrs. Henry C. Phipps, John H. Phipps, Mr. and Mrs. John S. Phipps and Michael Phipps.

**Snake venoms used in this study have been purchased from the South African Institute for Medical Research, Ross Allen's Reptile Institute, Fla. and the Miami Serpentarium, Fla. We are particularly indebted to Dr. P. Agerholm Christensen of the South African Institute for making available large supplies of *Naja flava* venom and for permitting us to study unpublished data on mouse toxicity studies carried out in his laboratory. He also kindly sent samples of the anavenom used in South Africa for production of antivenin.

***Tetanus and diphtheria toxoids were supplied by Eli Lilly and Co.

monkey interference tests. The greatest problem in the production of toxoids was their lack of stability after detoxication, but this has been overcome. No demonstrable fluctuation in stability over a 2-year period was noted in one of the standard products (40NF).

Viruses. The Brunhilde type of poliomyelitis obtained from Doctor David Bodian has been maintained as an emulsified pool of cervical and lumbar spinal cord enlargements from rhesus monkeys showing first signs of paralysis. This 1:5 emulsion in 20% rabbit serum in non-pyrogenic distilled water was distributed, quick-frozen and stored at -30°C. A titration of this pool yielded a PD_{50} of $10^{-3.7}$ in December, 1950. The specific nature of the strain was confirmed by a neutralization test with Brunhilde antiserum.* The Lansing strain was received from Dr. Charles Armstrong, U.S.P.H.S., and its nature was confirmed by neutralization test with serum from the same source as in the case of Brunhilde. This agent was handled as a mouse brain-cord pool harvested essentially in accordance with Ainslie's recommendations.[8] It yielded a mouse titer of $10^{-4.3}$. The method of preparation and storage was similar to that employed for the Brunhilde virus. Both strains of human poliomyelitis, in the course of this investigation, were inoculated into 6- to 8-lb. rhesus monkeys intracerebrally. The interference schedule was started 24 hours later via the subcutaneous route. In recording paralysis, animals were placed in 3 categories: (1) Negative (i.e., no gross sign of paralysis); (2) Non-prostrating paralysis (this varied from flaccidity of a single limb to heavy involvement of many muscle groups, but the animal could sit up and move about); (3) Prostrating paralysis (these animals either died or were killed 72 hours or more after appearance of quadriplegia). All experiments were followed for at least 30 days. The interference effect was evaluated on the basis of significant delay in the appearance of paralysis and/or by the number of survivors in groups receiving virus plus neurotoxoid compared to the control groups.

Controls. One or 2 control groups have been used in each experiment. In all instances, one control group received the same number of injections as in the lightest interference schedule, the control material consisting of buffer or 0.9% saline solution alone or with 1% alum (potassium aluminum sulfate) added, depending upon the toxoid solution used. Since November, 1950, the control injectable has consisted of the identical solution used in production of the toxoid *minus* the active principle, i.e., the toxin. Where second control groups were added, only virus was injected and the animals were not handled at any time after

*Received from Dr. Jonas Salk through the courtesy of the National Foundation for Infantile Paralysis.

the infecting dose was administered. The number of animals in each group within a given experiment has varied from 5 to 8 animals per group. Six monkeys per group are now routinely used.

Results. In the first positive interference experiment (November, 1949), 3 control animals developed non-prostrating or prostrating

TABLE 1

NAJA FLAVA TOXOID INTERFERENCE EFFECT, 24 HOURS AFTER INFECTION WITH 50 INTRACEREBRAL PD_{50} BRUNHILDE VIRUS IN *MACACUS RHESUS* (UNSTABLE TOXOIDS)

Control Groups				*Toxoid Groups*			
Prostrating Paralysis	Survivors			Prostrating Paralysis	Survivors		
	Total	Para-lyzed	Neg.		Total	Para-lyzed	Neg.
34/35*	1	0	1	116/154*	38	23	15
97.2%	2.8%		2.8%	75.3%	24.7%		9.7%

*No. prostrated animals/No. animals tested.

paralysis by the 7th day following intracerebral injection of approximately 50 PD_{50} of Brunhilde virus. They were quadriplegic by the 9th day. In contrast, 3 animals infected with the same sample of virus and at the same time as the controls, but receiving a subcutaneous injection of 3/8 ml/kg of *Naja flava* toxoid 24 hours later, showed no sign of paralysis until the 9th, 11th and 13th days. The comparative average incubation of 7 days for the controls as against 11 days for the animals receiving a single injection of toxoid seemed promising, although there were no survivors in this experiment. In later work, attempts were made to determine an interfering dose (I.D.) and to develop an interference schedule, since these factors are known to be important in demonstrating interference.[5,6,11] A toxoid dose range of 0.2 to 0.9 ml/kg bodyweight was tested under various circumstances and schedules of 1 to 20 injections of toxoid were studied in mice infected with Lansing and in monkeys infected with Brunhilde viruses. No significant data were obtained in the mouse experiments. In TABLE 1, the results of 4 interference experiments (Experiments 20, 21, 24, 25) 24 hours post infection in 189 rhesus monkeys have been summarized.

It must be emphasized that all animal groups in these experiments have been included, even though a number of the products were found to be unstable and injection schedules unsatisfactory. In spite of the inclusion of such negative groups, it will be seen that 38 (24.7%) of 154 monkeys in post 24 hour postinfection interference groups survived, as against one (2.8%) of 35 controls. Twenty-three of the 38 survivors were partially paralyzed. Fifteen of the 38 animals showed no gross sign of paralysis.

The interference effect was further studied when a stable toxoid became available (TABLE 2), A total of 9 I.D.'s of 3/8 ml/kg were administered in 4 fluid and 5 alum toxoid injections on the 1st, 2nd,

TABLE 2

NAJA FLAVA TOXOID INTERFERENCE EFFECT, 24 HOURS AFTER INFECTION WITH 50 INTRACEREBRAL PD_{50} BRUNHILDE VIRUS IN *MACACUS RHESUS* (STABLE TOXOIDS)

Exp. #33 Groups	Onset Paralysis		Prostrating Paralysis		Survivors		
	Animals	Days	Animals	Days	Total	Paralyzed	Neg.
Control	4/5*	7.75**	4/5*	9.5**	1	0	1
42NF***	6/6	13.0	4/6	15.25	2	2	0
200NF****	5/6	11.6	2/6	10.5	4	3	1

*Animals paralyzed/Total No. Animals in group.

**Avg. No. of days.

***Toxoid (1% *Naja flava* venom). LD_{50} in 20 g mice obtained by intravenous injection of 0.35 ml toxoid (saline q.s. 0.5 ml).

****Toxoid (2% *Naja flava* venom). LD_{50} in 20 g mice obtained by intravenous injection of 0.38 ml toxoid (saline q.s. 0.5 ml). Three parts toxoid diluted with one part saline before injection into monkeys.

3rd, 4th and 7th days after infection, with only an alum toxoid injection given on the 7th day. Fluid toxoid was injected subcutaneously into the right upper and lower quadrants of the abdominal area. Alum toxoid,* consisting of one fluid I.D. mixed with an equal volume of 1% alum (potassium aluminum sulfate) was injected in the opposite side. TABLE 2 shows significant delay in the appearance of paralysis in the interference groups. While the average number of days for control

*The term "alum precipitated toxoid" has not been used since evidence is not at hand to show that the detoxicated venom is actually concentrated by alum.

animals to become paralyzed was 7.75, comparable values in the two *Naja flava* toxoid groups were 13.0 and 11.6 and, with the 2% preparation (designated as 200NF), 4 of 6 survivors were noted as against one survivor in the control group.

When Experiment 33 (TABLE 2) was compared with earlier experiments, it was noted that the addition of a fluid toxoid injection on the 7th day improved the interference schedule. Therefore, in Experiment 34, 5 fluid and 5 alum toxoid I.D.'s were injected on the 1st, 2nd, 3rd, 4th and 7th days, making a total of 10 I.D.'s per monkey. Two groups of animals were also included in this experiment, receiving 2 additional I.D.'s (one fluid and one alum) on the 9th day. One of these groups was also given 2 I.D.'s on the 11th day. TABLE 3 suggests that the 10 I.D. schedule is the most effective for producing an interference effect since, of the 6 animals in that group, there were 5 survivors, 4 of them showing no gross sign of paralysis at the end of the 30-day observation period. It is also of interest that the 2-year old 40NF, made under standard conditions, interfered with infection sufficiently to permit 3 of the 6 monkeys to escape obvious paralysis. In the control group, only one severely paralyzed animal survived.

TABLE 3

NAJA FLAVA TOXOID INTERFERENCE EFFECT
24 HOURS AFTER INFECTION
WITH 50 INTRACEREBRAL PD_{50} BRUNHILDE VIRUS
IN *MACACUS RHESUS* (IMPROVED SCHEDULE
OF INJECTION WITH STABLE TOXOIDS)

Exp. #34 Groups	Onset Paralysis		Prostrating Paralysis		Survivors		
	Animals	Days	Animals	Days	Total	Paralyzed	Neg.
Control	6/6*	9.16**	5/6*	11.4**	1	1	0
42NF, 10 I.D.	2/6***	14.5	1/6	13.0	5	1	4
42NF, 12 I.D.	4/6	12.75	3/6	15.0	3	1	2
42NF, 14 I.D.	5/6	12.5	4/6	10.75	2	1	1
40NF, 10 I.D.	3/6	11.0	3/6	12.66	3	0	3

**M. rhesus* paralyzed (Total in group).

**Days average for presentation of paralysis and prostration.

***One of these animals showed transient, mild paralysis on 16th day, with apparent recovery by 22nd day.

Since both 42NF* and 40NF* toxoids were used in Experiment 34, mention should be made of their residual toxicity. Toxoid 42NF is a preparation of 1% *Naja flava* venom** detoxicated to the point where 0.35 ml of the toxoid, diluted to a volume of 0.5 ml with saline, produced an LD_{50} in 20 g. mice following intravenous injection. In the case of 40NF, it is necessary to inject mice with 0.55 ml of the toxoid before an LD_{50} can be obtained.

Naja flava toxoid interference with Lansing virus. Although *Naja flava* toxoid had shown no interfering effect against Lansing infection in mice, the problem was re-examined in rhesus monkeys, in the light of results obtained in Experiment 34. The same technique used in Experiment 34 was followed in the Lansing experiment, i.e., 5 fluid and 5 alum toxoid injections constituted the interference schedule on the 1st, 2nd, 3rd, 4th and 7th days post infection. A PD_{50} for monkeys cannot be given since the Lansing virus has been maintained in mice with an LD_{50} of $10^{-4.3}$. The 2 groups of monkeys recorded in TABLE 4 were injected intracerebrally with 0.65 ml of a 20% suspension of the Lansing pool. From previous experience, it was known that this Lansing pool produced a characteristic and severe disease in monkeys following intracerebral injection.

The results in TABLE 4 confirm the adequacy of the interference technique as developed in the Brunhilde experiments. Although the first paralysis occurred in the interference group on the 6th day, the majority of controls (5 out of 6 animals) succumbed to severe paralysis by the 9th day, no additional interference monkeys showing paralysis until the 11th day. At the end of the 30-day period, only one control remained, while four of the interference monkeys were grossly free of paralysis.

Two experiments now in progress in which the experimental conditions are the same as those noted in the first Lansing experiment tend to confirm the results seen in TABLE 4.

Diphtheria and tetanus toxoids. Diphtheria and tetanus toxoids have been studied in 4 experiments for their interfering effect. Two of the experiments were under the conditions described in this report. Of 12 monkeys tested, none survived for 30 days. In one experiment, the average incubation period for appearance of paralysis was 2 days longer than in the control group. This was not considered significant, particularly in view of the results obtained with the *Naja flava* toxoid.

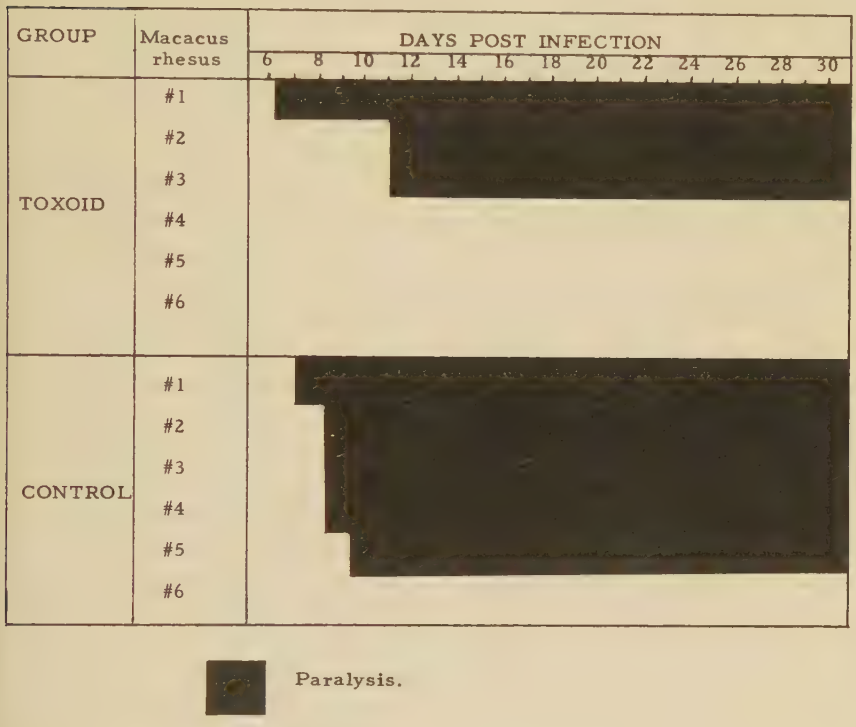
Naja naja toxoid interference. Five experiments have been carried

*Approximately 700 ml of 42NF and 40NF are available for investigative purposes.

**Titrations in mice, with the pool of *Naja flava* venom used as a source for 40NF and 42NF, have yielded LD_{50}'s of approximately 12 gammas.

TABLE 4

NAJA FLAVA TOXOID INTERFERENCE EFFECT, 24 HOURS AFTER LANSING VIRUS INTRACEREBRAL INFECTION IN *MACACUS RHESUS* (STABLE TOXOID).



out with *Naja naja* toxoid in rhesus monkeys infected intracerebrally with Brunhilde virus. On 2 occasions, the experimental conditions coincided with other experiments in the present report. The increase in average number of days before onset of paralysis in the *Naja naja* injected animals was 3 and 4 days more than in the control groups. There were also 1 and 2 survivors in these 2 groups, against no control survivors. In the 3 other experiments which are not discussed here, the trend suggested that this venom, detoxicated in a manner similar to *Naja flava*, produced an interfering effect. Since the original work of Lamb and Hunter[1,2] was done with *Naja naja* venom and its action, so far as we have been able to determine, does not differ significantly from *Naja flava* toxin, it would appear that the products of the two species of cobra have a basically similar effect.

Discussion. Lamb and Hunter,[1,2] in 1904, emphasized the neuro-

toxic character of Indian cobra venom by injecting it into rhesus monkeys and demonstrating a spectrum of pathologic alterations from chromatolysis to neuronophagia, the most marked effect occurring at the lumbar levels of the spinal cord. The possibility that detoxication of venom would reduce or destroy undesirable components without affecting neurotropic properties was the initial rationale for this investigation of neurotoxoid interference in experimental poliomyelitis. The interfering capacity of *Naja flava* toxoids as demonstrated in this study suggests that the neurotropic action has indeed been retained. This has been confirmed by histopathologic changes seen in motoneurons following subcutaneous administration of massive doses of the toxoids.[11] Since both toxin and toxoid produce chromatolysis, our thesis has been extended by correlation of data assembled by investigators in studies not related to this work but concerned with enzymatic changes associated with chromatolysis and with the enzymatic activity of cobra neurotoxin (cobra venom heated to 100°C, 15 minutes).

Following nerve amputation, chromatolysis occurs in the regional motor cells. In these cells, coincidental with a period of resistance to poliomyelitis infection,[12] there is a maximum inhibition of succinic dehydrogenase[13] activity and a decrease in cytochrome oxidase.[15] Similarly, following administration of *Naja flava* neurotoxoid, chromatolysis occurs and a poliomyelitis refractory period ensues, presumably associated with a decrease in both succinic dehydrogenase and cytochrome oxidase. The statement relating to the action of the cobra toxoid on enzymes is based on the report of Braganca and Quastel[14] who investigated heated cobra neurotoxin effect on various enzyme systems and showed its inhibiting action on the succinic dehydrogenase and cytochrome oxidase of brain tissue. In view of the similar behavior of venom toxoid and toxin in our hands, the assumption appears to be justified in attributing enzymatic action of the heated toxin to our toxoid.

The foregoing attempt to introduce a preliminary explanation for the neurotoxoid interference phenomenon is simply an application of the results of current experiments to accepted data and certainly does not imply a final solution to the problem, neither does it preclude additional mechanisms. Enzyme systems other than those mentioned have been involved in studies of regenerating neurons but have not been discussed and no attempt has been made to correlate the role of nucleoproteins in regenerating or resistant motoneurons.

Conclusions. (1) An interference phenomenon has been demonstrated by the use of *Naja flava* and *Naja naja* toxoids injected into rhesus

monkeys 24 hours after intracerebral infection with either Brunhilde or Lansing poliomyelitis viruses. (2) No interference activity has been demonstrated in mice infected with Lansing virus. (3) An attempt has been made to explain the interference activity by the neurotoxoids, correlating known experimental results of enzymatic action in motoneurons refractory to poliomyelitis infection with the action of heated cobra venom.

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